American Journal of Analytical Chemistry, 2011, 2, 352-362
doi:10.4236/ajac.2011. 23043 Published Online July 2011 (http://www.scirp.org/journal/ajac)

A Validated Liquid Chromatography-Mass Spectrometry Method for the Detection and Quantification of Oxidative Metabolites of 2,2',4,4'-Tetrabromodiphenyl Ether in Rat Hepatic Microsomes

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Received January 5, 2011; *revised March* 4, 2011; *accepted March* 15, 2011

Abstract

In the present study, we developed and validated an analytical method using ultra performance liquid chromatography-mass spectrometry (UPLC/MS) for the quantitative determination of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) metabolism by rat hepatic microsomes. BDE-47 is a brominated flame retardant that was widely used in a variety of consumer products and has subsequently been identified as a ubiquitous environmental contaminant. Hydroxy-bromodiphenyl ethers (OH-BDEs) were isolated from rat hepatic microsomes by liquid-liquid extraction. Chromatographic separation was achieved by UPLC on a C_{18} column with gradient elution using a mobile phase consisting of methanol and water, each containing 0.1% formic acid, at a flow rate of 0.2 mL/min. Detection and quantification were performed using a mass spectrometer in single ion recording mode with negative electrospray ionization. The UPLC/MS method was validated for linearity, limit of quantification (LOQ), accuracy, precision and recovery. The weighted calibration curves ($1/X^2$) were linear over a concentration range of 5 - 250 nM with LOQ values between 5 nM and 50 nM for the individual OH-BDEs. Intra- and inter- day accuracy (%DEV) and precision (%RSD) values ranged from –11.7% to 9.5% and 5.9% to 16.5%, respectively. Recovery values of 70% to 90% were obtained for all OH-BDEs. The validated method allowed us to successfully analyze metabolite formation following incubation of BDE-47 with hepatic microsomes prepared from phenobarbital-treated rats. Results demonstrate that the UPLC/MS method has sufficient sensitivity and reproducibility to fully characterize the *in vitro* metabolism of BDE-47 and possibly other PBDEs.

Keywords: BDE-47, Hepatic Metabolism, Polybrominated Diphenyl Ethers, Rat Hepatic Microsomes, Ultra Performance Liquid Chromatography-Mass Spectrometry.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are halogenated aromatic hydrocarbons that have been used as additive flame retardants on a variety of consumer products since 1965 [1]. PBDEs were marketed as commercial mixtures containing a limited number of the 209 possible brominated diphenyl ether (BDE) congeners [2]. The penta-BDE mixture, which was used extensively in North America [3], was composed predominantly of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), 2,2',4,4',5-pentabromodiphenyl ether (BDE-99), 2,2',4,4',6-pentabromodiphenyl ether (BDE-100), 2,2',4,4',5,5'-hexabromo- diphenyl ether (BDE-153) and 2,2',4,4',5,6'-hexabromo- diphenyl ether (BDE-154) [4]. Penta-BDE was applied to epoxy resins, textiles, paints and flexible polyurethane foam, which was used in upholstered furniture, mattresses and carpet padding [5,6]. PBDEs are not chemically bound to the polymer components of the products to which they are applied and can be released into the environment during manufacture [5], use [7] and disposal [8] of these products. This factor, together with the high lipophilicity, chemical stability and the widespread use of BDE mixtures has resulted

in the ubiquitous distribution of PBDEs in the environment [1]. BDE-47, for example, has been detected in air [9], sediment [10], fish [10,11], marine mammals [12,13] and in human blood [14], adipose tissue [15] and breast milk [16] and is frequently the predominant PBDE congener found in biotic samples [1]. Studies with laboratory animals have shown that developmental exposure to BDE-47 caused alterations in neuromotor activity [17] and exposure *in utero* produced changes to the reproductive system and thyroid gland of female rat pups [18].

Laboratory studies have shown that PBDEs can be metabolized by hepatic cytochrome P450 enzymes to hydroxy-BDEs (OH-BDEs) [19-22]. OH-BDEs are of toxicological interest as OH-BDEs show a greater affinity for the thyroid hormone receptor than the natural ligand or PBDEs themselves [19]. OH-BDEs have been detected in blood and feces of rodents treated with BDE-47 [21] and various OH-BDEs have been found in human plasma [23]. Several studies, including those mentioned above, examined the formation of OH-BDEs; however, the specific enzymes and enzyme kinetics of OH-BDEs formation are poorly understood. Characterization of BDE-47 metabolism *in vitro* is needed to develop a better understanding of the role of metabolism in the bioaccumulation and toxicity of BDE-47.

The most common method for the detection and quantification of PBDEs and OH-PBDEs in environmental samples has been gas chromatography-mass spectrometry (GC/MS) or gas chromatography coupled with electron capture detection (GC/ECD) [19]. In a study that examined the endocrine disrupting activity of BDEs following hepatic biotransformation, Hamers *et al*. [19] identified six hydroxylated metabolites of BDE-47 using a GC/MS method. GC-based methods are sensitive, but require derivatization of OH-BDEs, additional sample preparation time, the use of harmful derivatizing agents and possible underestimation of OH-BDE concentrations due to incomplete derivatization. Liquid chromatography coupled with mass spectrometry (LC/MS) provides an alternative analytical technique that does not require derivatization. Mas *et al.* demonstrated that LC/MS can be used to detect and quantify OH-BDEs in soil, fish, sludge and particulate matter that was spiked with a mixture of OH-BDEs [24]. However, their LC/MS method was not validated in a biological matrix [24] and its applicability to the detection and quantification of oxidative metabolites of BDE-47 generated *in vitro* or *in vivo* is unknown.

The aim of the present study was to develop and validate a UPLC/MS-based analytical method to detect and quantify OH-BDEs and apply this method to investigate the *in vitro* biotransformation of BDE-47 by rat hepatic microsomes.

2. Materials and Methods

2.1. Chemicals and Reagents

BDE-47 (neat, 99% purity) was obtained from Chiron (Trondheim, Norway). 4'-Hydroxy-2,2',4-tribromodiphenyl ether (4'-OH-BDE-17), 2'-hydroxy-2,4,4'-tribromodiphenyl ether (2'-OH-BDE-28), 4-hydroxy-2,2',3,4'-tetrabromodiphenyl ether (4-OH-BDE-42), 3-hydroxy-2, 2',4,4'-tetrabromodiphenyl ether (3-OH-BDE -47), 5-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (5-OH-BDE-47), 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH-BDE-47), 4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether (4'-OH-BDE-49) (10 μg/mL or 50 μg/mL in acetonitrile, purity of at least 98%) and 4'-hydroxy-2,2',4,6'-tetrachlorobiphenyl (4'-OH-CB-50, neat, 99% purity) were purchased from AccuStandard (New Haven, Connecticut, USA). Magnesium chloride, sucrose and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Methanol, methyl-*tert*-butyl ether, hexane, isopropanol, sodium hydroxide, hydrochloric acid and mono- and di-basic potassium phosphate were purchased from Fisher Scientific (Ottawa, Ontario, Canada). Hydrochloric acid and all organic solvents were HPLC- grade or higher. Ultra pure water was obtained using a Milli-Q Synthesis system (Millipore, Billerica, MA, USA).

2.2. Rat Hepatic Microsomes

Adult male Long Evans rats (body weight between 160-190 g) were purchased from Charles River Laboratories (Montreal, PQ, Canada). Rats were cared for in accordance with the principles and guidelines outlined by the Canadian Council of Animal Care. Rats ($n = 6$) were treated with sodium phenobarbital (PB, 80 mg/kg/day) for 3 days, as previously described by Edwards *et al.* [25]. Pooled hepatic microsomes were prepared by differential centrifugation as previously described [26]. Hepatic microsomes were aliquoted and stored at –80˚C until use. Protein concentration was determined by the method of Lowry, *et al.* with bovine serum albumin as the standard [27].

2.3. Standard Solutions

A stock solution of BDE-47 at a concentration of 2.5 mM was prepared in methanol. A stock solution of OH-BDE standards containing 4'-OH-BDE-17, 2'-OH-BDE-28, 4-OH-BDE-42, 3-OH-BDE-47, 5-OH-BDE-47, 6- OH-BDE-47 and 4'-OH-BDE-49 (at 1.25 μM each) was prepared in methanol. A second

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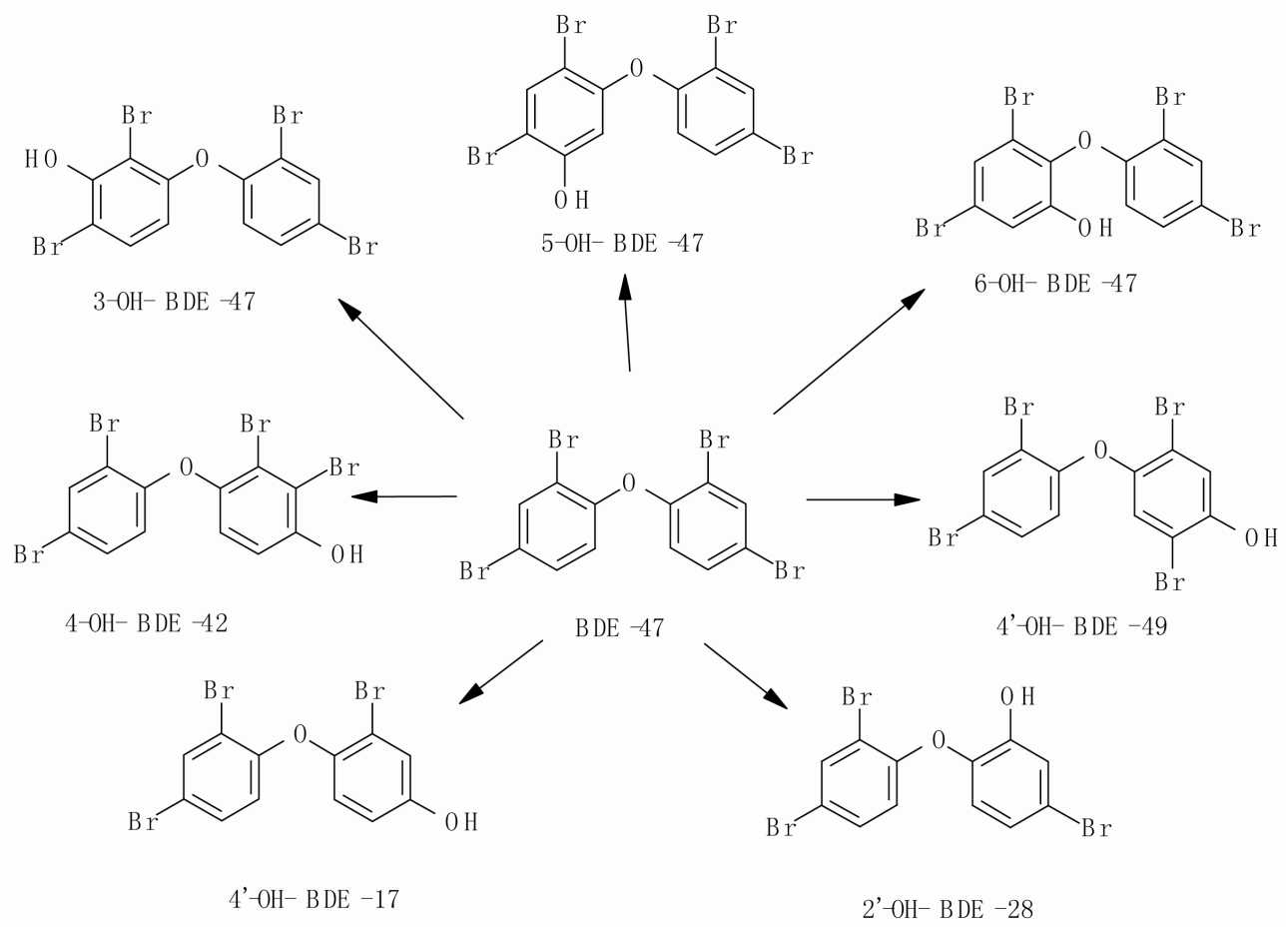


Figure 1. Chemical structures of BDE-47 and seven possible hydroxy-metabolites.

stock solution of OH-BDE standards at 0.125 μM each was prepared by diluting an aliquot of the first stock solution 10-fold in methanol. A stock solution of internal standard containing 4'-OH-CB-50 at a concentration of 125 μM was prepared in methanol. Three quality control (QC) solutions containing OH-BDE standards at concentrations of 7.5, 37.5 and 175 nM each and internal standard at a concentration of 1.25 μM were prepared in methanol. These QC solutions were used for system suitability tests and recovery determination. All stock solutions were stored in amber vials at –20˚C until needed and each vial was vigorously vortex-mixed before use.

2.4. Sample Preparation

Stock solutions of OH-BDE standards were spiked into rat hepatic microsomes to prepare calibration standards (CS) samples for the generation of calibration curves. CS samples were prepared by mixing 1 mg of hepatic microsomal protein, 50 mM potassium phosphate buffer containing 3 mM magnesium chloride (pH 7.4) and an appropriate volume of OH-BDE stock solution in a final volume of 1 mL. Final concentrations of the OH-BDE standards in the CS samples were 2.5, 5, 10, 25, 50, 100 and 250 nM. QC samples at three concentrations were prepared in the same manner. Final concentrations of OH-BDE standards in the QC samples were 7.5, 37.5 and 175 nM (low, medium and high, respectively). The QC samples were used for the determination of accuracy and precision. Blank samples containing only hepatic microsomal protein and phosphate buffer were also prepared.

CS, QC and blank samples were incubated for 5 min at 37˚C in a shaking water bath. Following incubation, 1 mL of ice-cold 0.5 M sodium hydroxide was added, and each tube was immediately vortex-mixed. An aliquot of internal standard (10 μL of 125 μM) was added to each tube. Tubes were capped and placed in a 70˚C water bath for 10 min. After cooling to room temperature, 2 mL of 6 M hydrochloic acid and 1 mL of isopropanol

was added to each tube. Tubes were then vigorously vortex-mixed for 1 min. Two mL of a methyl-*tert*-butyl ether: hexane (1:1 *v/v*) mixture was then added to each

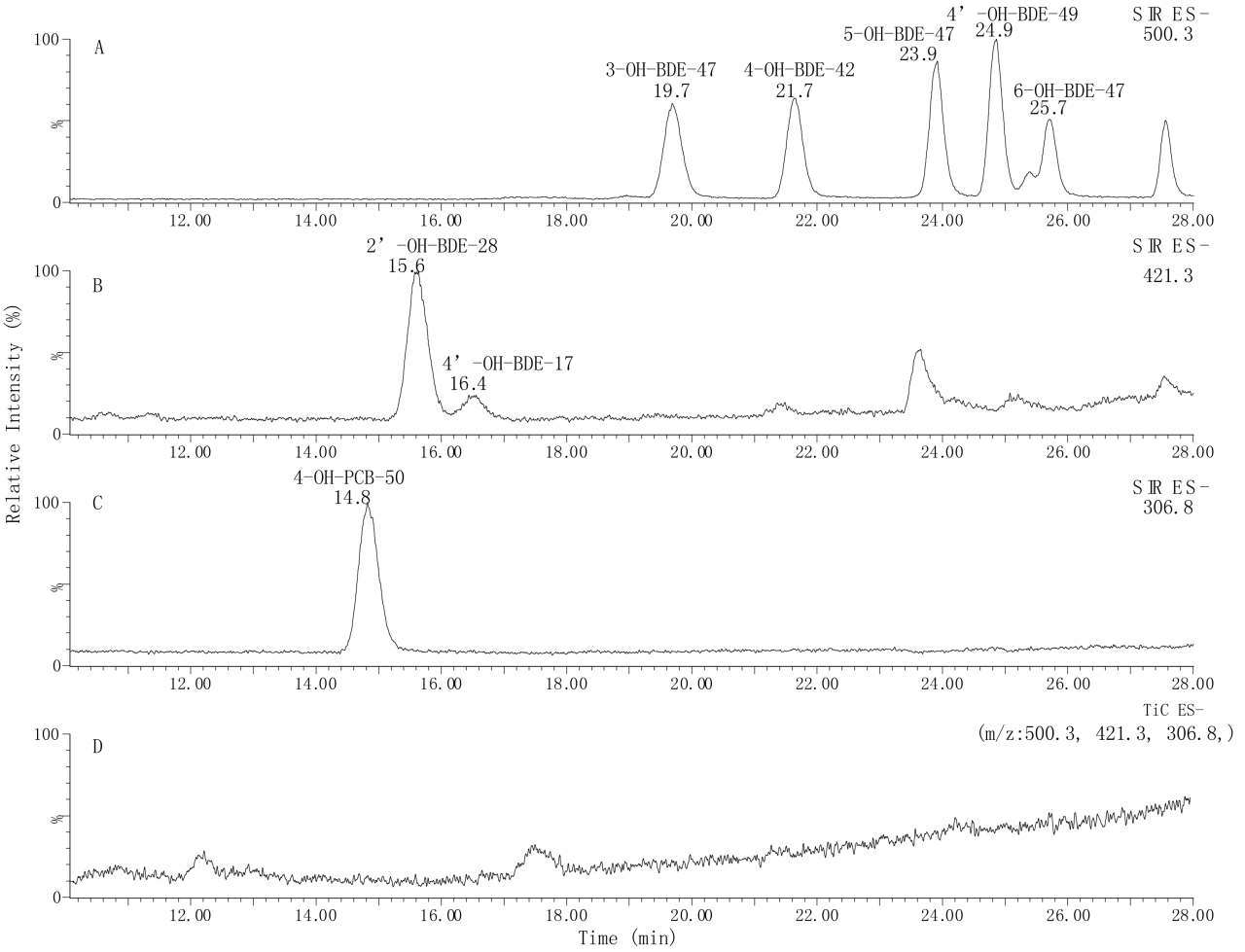


Figure 2. Representative UPLC/MS ion chromatograms showing peaks obtained by spiking rat hepatic microsomes (at a concentration of 1 mg/mL) with a calibration standard stock solution (final concentration of OH-BDE standards was 50 nM) and the internal standard solution (final concentration was 1.25 µM. (a) Chromatogram of OH-tetra BDEs standards (3-OH-BDE-47, 4-OH-BDE-42, 5-OH-BDE-47, 4'-OH-BDE-49 and 6-OH-BDE-47) at *m/z* 500.3; (b) chromatogram of OH-tri-BDE standards (2'-OH-BDE-28 and 4'-OH-BDE-17) at *m/z* 421.3; (c) chromatogram of internal standard (4-OH-PCB-50) at *m/z* 306.8; (d) total ion current of a blank sample.

tube. Tubes were vigorously vortex-mixed for 1 min and spun in a centrifuge at 2,500 rpm for 5 min. The top organic layer was carefully removed and transferred into clean test tubes. The extraction procedure was repeated two more times. The organic phase from each extraction of the same sample was pooled and evaporated under a gentle flow of nitrogen. The residue was reconstituted in 250 µL of methanol, vortex-mixed and filtered through a syringe filter (polytetrafluoroethylene membrane, 0.45 µM) into a 300-µL HPLC vial.

2.5. UPLC/MS Conditions

The UPLC/MS system consisted of a Waters Acquity UPLC Sample Manager and a Waters Acquity UPLC Binary Solvent Manger connected to a Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with a combined Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) probe (Waters, Milford, MA, USA). Chromatographic separation was achieved using a Waters Acquity UPLC BEH C_{18} (2.1 × 100 mm, 1.7 µm) column, which was maintained at 50ºC. The autosampler tray was maintained at 10ºC and the injection volume was 5 µL. The mobile phase consisted of water containing 0.1% (*v/v*) formic acid (solvent A) and methanol containing 0.1% (*v/v*) formic acid (solvent B). Mobile phase solvents were filtered through a membraine filter (Millipore Durapore

Membrane Filters, 0.22 μm GV, Billerica, USA) prior to use. The gradient program was 35% solvent A and 65% solvent B from 0 to 15 min followed by a linear increase to 80% solvent B from 15 to 30 min. At 30.1 min, solvent B was increased to 100% and maintained for 5 min. The column was then re-equilibrated with 35% solvent A and 65% solvent B for 5 min. The flow rate was maintained at 0.2 mL/min and the total analysis time was 40 min. To protect the mass spectrometer from contamination, the mobile phase flow was diverted to waste between 0 and 10 min and 30 and 40 min of each injection.

Table 1. Limit of quantification (LOQ) of individual OH-BDE standards.

Authentic Standard	LOQ (nM)	Mean Measured Concentration (nM)	Accuracy (%Dev)	Precision (%RSD)	S/N
4'-OH-BDE-17	50	42.9 ± 6.5	–14.2	15.1	5.4
2'-OH-BDE-28	5	5.5 ± 0.6	10.0	10.9	4.9
4-OH-BDE-42	10	11.3 ± 0.9	13.0	8.0	4.9
3-OH-BDE-47	10	10.7 ± 1.2	7.0	11.2	4.9
5-OH-BDE-47	5	5.6 ± 0.3	12.0	5.4	3.5
6-OH-BDE-47	10	10.8 ± 1.9	8.0	17.5	4.1
4'-OH-BDE-49	5	5.6 ± 0.4	12.0	7.1	3.9

n=6, mean ± SD

The mass spectrometer was operated in negative electrospray ionization mode (ESI) using selected ion recording (SIR) at a capillary voltage of 3 kV, cone voltage of 40 V, source temperature of 120ºC, desolvation temperature of 400ºC and desolvation gas flow of 1005 L/h. The UPLC/MS system was controlled by MassLynx v. 4.1 software and Windows XP operating system. Chromatographic peaks corresponding to the OH-BDEs and the internal standard were identified by comparing the mass-to-charge ratio (*m/z*) and retention time values with those of the authentic standards: *m/z* 421.3 for 4'-OH-BDE-17 and 2'-OH-BDE-28; *m/z* 500.6 for 4-OH-BDE-42, 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47 and 4'-OH-BDE-49; and *m/z* 306.7 for 4'-OH-CB-50.

2.6. Method Validation

The UPLC/MS method was validated for accuracy, precision, linearity, limit of quantification (LOQ), selectivity and recovery. Accuracy and precision were assessed using the three QC samples. Accuracy was calculated using the mean measured concentration and expressed as percent deviation (%Dev) of the nominal concentration. Precision was expressed as percent relative standard deviation (%RSD). To determine intra-day accuracy and precision, six replicates of each QC sample were prepared and analyzed on the same day. To determine inter-day accuracy and precision, QC samples were prepared and analyzed in triplicate on three separate days. The acceptance criteria for inter- and intra- day accuracy and precision were %Dev ± 20% and %RSD ≤ 20%, respectively, for the low QC samples and %Dev ± 15% and %RSD ≤ 15%, respectively, for the medium and high QC samples.

Linearity of the calibration curve was assessed using the coefficient of determination (R^2). Calibration curves were generated for each OH-BDE standard by plotting peak area ratios for each OH-BDE standard and internal standard (y-axis) against the corresponding nominal concentration of the OH-BDE standard (nM, X-axis) using linear regression analysis. To increase accuracy at the lower end of the calibration curve, a $1/X^2$ weighting factor was used. The acceptance criterion for linearity was $R^2 \geq 0.9$.

The LOQ of each OH-BDE standard was determined by preparing five replicates of CS samples between 2.5 and 50 nM and a calibration curve. The LOQ was set as the lowest CS concentration that had a signal-to-noise (S/N) ratio at least three times higher than that of the

blank sample with a ± 20% Dev and ≤ 20% RSD. The S/N ratio was determined with MassLynx using the peak-to-peak method.

Selectivity was assessed by visually comparing the chromatograms obtained from blank samples with chromatograms from spiked CS samples at the LOQ value of each OH-BDE standard. Chromatograms were examined for the presence of interfering peaks with retention times that overlapped those of OH-BDE standards or the internal standard.

Recovery rates were determined using QC solutions and QC samples. Recovery was calculated by comparing the peak area of each OH-BDE standard in the QC sample with that in the QC solution at the same concentration.

Table 2. Intra-day accuracy (%Dev) and precision (%RSD).

Metabolite	Nominal Concentration (nM)	Mean Measured Concentration (nM)	Accuracy (%Dev)	Precision (%RSD)
4'-OH-BDE-17	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	< LOQ	n. d.	n. d.
	QC-High (175)	167.0 ± 17.7	–4.6	10.6
2'-OH-BDE-28	QC-Low (7.5)	n. d.	n. d.	n. d.
	QC-Med (37.5)	36.1 ± 3.3	–3.7	9.1
	QC-High (175)	162.3 ± 15.7	–7.3	9.7
4-OH-BDE-42	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	36.7 ± 2.2	–2.1	5.9
	QC-High (175)	153.9 ± 15.4	–12.1	10.0
3-OH-BDE-47	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	36.8 ± 2.8	–2.1	7.6
	QC-High (175)	156.2 ± 14.9	–10.7	9.5
5-OH-BDE-47	QC-Low (7.5)	8.2 ± 1.4	9.3	17.0
	QC-Med (37.5)	36.6 ± 3.0	–2.4	8.2
	QC-High (175)	155.7 ± 15.3	–11.0	9.8
6-OH-BDE-47	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	38.3 ± 3.0	2.1	7.8
	QC-High (175)	178.2 ± 19.1	1.8	10.7
4'-OH-BDE-49	QC-Low (7.5)	7.8 ± 0.5	4.0	6.4
	QC-Med (37.5)	35.8 ± 3.2	–4.5	8.9
	QC-High (175)	157.3 ± 13.9	–10.1	8.8

< LOQ = Below limit of quantification; n .d., not determined because metabolite concentration was below the LOQ; n = 6, mean ± SD.

2.7. In Vitro Biotransformation of BDE-47

Following validation, the UPLC/MS method was applied to investigate the *in vitro* biotransformation of BDE-47. Formation of hydroxy BDE-47 metabolites by rat hepatic microsomes was determined using a reaction mixture containing 50 mM phosphate buffer, 0 - 2 mg/mL of rat hepatic mirosomal protein and 50 μM BDE-47 (20 μL of 2.5 mM in methanol) in a final volume of 0.99 mL. After a 5-min pre-incubation at room temperature, the reaction was initiated by addition of 10 μL of 100 mM NADPH

(final concentration 1 mM) and allowed to proceed at 37˚C in a shaking water bath for 0 to 30 min. The reaction was terminated by the addition of 1 mL of ice-cold 0.5 M sodium hydroxide. Extraction and quantification of the OH-BDE metabolites was performed as described above. Samples were prepared in duplicate for each assay and experiments were performed 3 times on separate days. Blank samples contained only rat hepatic microsomes and buffer. Negative control samples did not contain BDE-47, NADPH, or rat hepatic microsomes. QC samples were included in each experiment to assess method performance.

Table 3. Inter-day accuracy (%Dev) and precision (%RSD).

Metabolite	Nominal Concentration (nM)	Mean Measured Concentration (nM)	Accuracy (%Dev)	Precision (%RSD)
4'-OH-BDE-17	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	< LOQ	n. d.	n. d.
	QC-High (175)	164.3 ± 22.3	–6.1	13.6
2'-OH-BDE-28	QC-Low (7.5)	n. d.	n. d.	n. d.
	QC-Med (37.5)	37.3 ± 4.8	–0.4	12.7
	QC-High (175)	158.4 ± 12.0	–9.5	7.6
4-OH-BDE-42	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	37.8 ± 4.0	0.8	10.6
	QC-High (175)	155.4 ± 11.5	–11.2	7.4
3-OH-BDE-47	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	37.6 ±3.0	0.3	10.1
	QC-High (175)	155.2 ± 11.5	–11.3	7.4
5-OH-BDE-47	QC-Low (7.5)	6.9 ±2.5	–8.0	8.0
	QC-Med (37.5)	37.4 ±4.2	–0.3	11.3
	QC-High (175)	156.3 ± 13.0	–10.7	8.3
6-OH-BDE-47	QC-Low (7.5)	< LOQ	n. d.	n. d.
	QC-Med (37.5)	39.5 ± 4.3	5.3	10.9
	QC-High (175)	182.8 ± 14.8	4.4	8.1
4'-OH-BDE-49	QC-Low (7.5)	7.7 ± 0.5	2.7	6.5
	QC-Med (37.5)	35.7 ± 4.8	–4.8	13.4
	QC-High (175)	154.6 ± 11.0	–11.7	7.1

< LOQ = Below limit of quantification; n. d., not determined because metabolite concentration was below the LOQ; n = 12 (Day 1 n = 6, Day 2 n = 3, Day 3 n. = 3) mean ± SD.

3. Results and Discussion

3.1. Optimization of UPLC/MS Parameters

APCI and ESI in negative and positive modes were compared for the detection of OH-BDE standards by mass spectrometry using SIR. OH-BDE standards were not detected in positive ion mode. ESI operated in negative electrospray ion mode produced larger peak area counts than APCI. BDE-47 was not detected with APCI

or ESI in either positive or negative ion mode. Flow injection analysis was used to determine the molecular ions $[M-H]^-$ and optimal cone voltage (40 V) for all OH-BDE standards. A source temperature of 120˚C, a desolvation temperature of 400˚C and a desolvation gas flow of 1005 L/H were found to be optimal for the detection of OH-BDE standards. Multiple reaction monitoring (MRM) was also assessed. Product ion scans of the molecular ions were performed at different collision energy values. The main product ion was a bromine fragment (*m/z* 79 and *m/z* 81). However, the sensitivity of the MRM method was much lower than that of the SIR method. Therefore, SIR was used for subsequent analyses.

Table 4. Recovery values of individual OH-BDE standards.

Authentic Standard	Retention Time (min)	Nominal Concentration (nM)	Mean Recovery (%)
4'-OH-BDE-17	16.4	QC-Low (7.5)	n. d.
		QC-Med (37.5)	n. d.
		QC-High (175)	69.9 ± 5.6
2'-OH-BDE-28	15.6	QC-Low (7.5)	n. d.
		QC-Med (37.5)	86.7 ± 5.3
		QC-High (175)	72.3 ± 4.3
4-OH-BDE-42	21.7	QC-Low (7.5)	
		QC-Med (37.5)	84.0 ± 6.0
		QC-High (175)	74.0 ± 5.3
3-OH-BDE-47	19.7	QC-Low (7.5)	n. d.
		QC-Med (37.5)	88.0 ± 4.4
		QC-High (175)	76.7 ± 5.0
5-OH-BDE-47	23.4	QC-Low (7.5)	89.2 ± 6.2
		QC-Med (37.5)	88.2 ± 4.6
		QC-High (175)	75.2 ± 4.7
6-OH-BDE-47	25.7	QC-Low (7.5)	n. d.
		QC-Med (37.5)	88.2 ± 4.3
		QC-High (175)	74.0 ± 4.9
4'-OH-BDE-49	24.9	QC-Low (7.5)	87.6 ± 5.3
		QC-Med (37.5)	87.6 ± 4.2
		QC-High (175)	75.4 ± 5.1

n. d., not determined because metabolite concentration was below the LOQ; n=6, mean ± SD

Separation of the seven OH-BDE standards was achieved using a Waters Acquity UPLC BEH C_{18} (2.1 × 100 mm, 1.7 µm) column and gradient elution. Several mobile phase combinations were tested, including mixtures of water and acetonitrile or water and methanol. A mixture of water containing 0.1% formic acid (*v/v*) and methanol containing 0.1% formic acid (*v/v*) yielded the best peak shape and the highest peak area counts and was selected for chromatographic separation. Formic acid was added to enhance ionization of the compounds of interest. Isocratic and gradient elution were also compared. The best resolution was achieved using the elution gradient program reported in the experimental section, which gave baseline separation of all OH-BDE standards tested, except for 6-OH-BDE-47 and 4'-OH-BDE-49 (**Figure 2**). Various elution gradient programs, flow rates

and run times were tried but complete baseline separation of 6-OH-BDE-47 and 4'-OH-BDE-49 was not achieved. In comparison, Mas, *et al.*, [25] used a ternary mixture of ammonium acetate, acetonitrile and methanol, gradient elution and a shorter run time (20 min compared to 40 min in the present study) to resolve eight OH-BDEs but did not attain baseline separation of 5-OH-BDE-47, 6-OH-BDE-47 and 4'-OH-BDE-49 or of three hydroxylated tribrominated diphenyl ethers.

3.2. Optimization of Sample Preparation

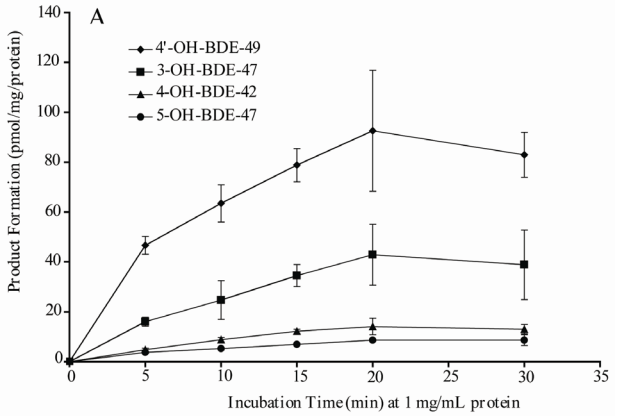
To achieve efficient extraction of the OH-BDE standards from the biological matrix (*i.e.*, rat hepatic microsomes) and to minimize the possibility of unknown peaks that could interfere with the peaks of interest, variations of the sample preparation protocol were evaluated. Hexane, acetone, dichloromethane, methyl-*tert*-butyl ether, as well as, different ratios of acetone and hexane and of methyl-*tert*-butyl ether and hexane were tested as possible extraction solvents. Three extractions with a mixture of methyl-*tert*-butyl ether: hexane (1:1 *v/v*) yielded the highest recovery of the OH-BDE standards (**Table 4**). The addition of a centrifugation step before extraction, to separate microsomes from supernatant, or replacement of sodium hydroxide with acetone, methanol, formic acid, hydrochloric acid, sulfuric acid or trifluroacetic acid, to terminate the reaction, were tested. Although some of these modifications reduced the appearance of unknown non-interfering peaks in the blank samples, the modifications reduced the recovery of the OH-BDE standards and were not incorporated into the assay.

4. Method Validation

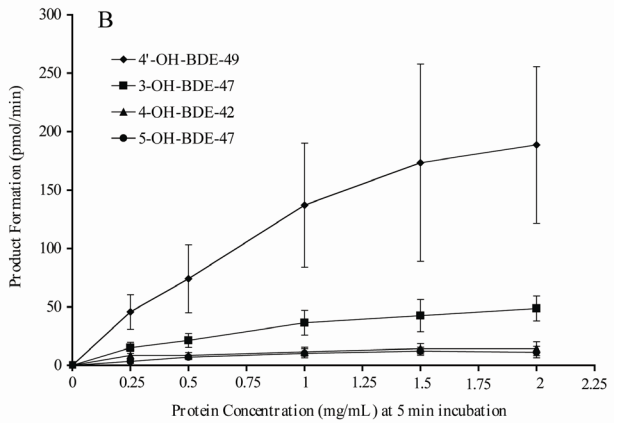
Visual inspection of chromatograms obtained from blank samples and chromatograms obtained from spiked CS samples showed no interfering peaks at the same *m/z* and retention times as the OH-BDE standards or internal standard. Representative chromatograms obtained from a CS sample at 50 nM and a blank sample are shown in **Figure 2**.

Mean R^2 values of the calibration curve constructed for each individual metabolite were 0.95 ± 0.06 for 4'-OH-BDE-17, 0.96 ± 0.04 for 2'-OH-BDE-28, 0.94 ± 0.05 for 4-OH-BDE-42, 0.96 ± 0.04 for 3-OH-BDE-47, 0.97 ± 0.04 for 5-OH-BDE-47, 0.94 ± 0.06 for 6-OH-BDE-47 and 0.95 ± 0.05 for 4'-OH-BDE-49. The LOQ concentration of individual OH-PBDEs ranged between 5 and 50 nM (**Table 1**). The LOQ concentrations determined in this study, which are in the pg/mL range, are similar to those reported by Erratico, *et al*., for OH-penta-BDEs using a similar UPLC/MS method [28] and comparable to those reported by Mas, *et al.* [24], using LC/MS ion spray operated in negative ion MRM mode.

Intra- and inter-day accuracy and precision values are reported in **Tables 2** and **3**, respectively. Both intra- and inter-day accuracy and precision fell within the established acceptance criteria. In contrast to the study by Mas, *et al*. [24], our experimental design evaluated accuracy and precision using the biological matrix of interest and concentrations at the low, medium and high range of the calibration curve rather than at a single point in an organic solvent (*i.e.*, 75 pg/ml, approximately 150 nM). Evaluation using the biological matrix and multiple points on the calibration curve provides a more robust estimation of accuracy and precision.



(a)



(b)

Figure 3. Effect of (a) incubation time and (b) microsomal protein concentration on OH-BDE metabolite formation. BDE-47 (50 μM) was incubated with hepatic microsomes prepared from phenobarbital-treated adult male rats for (a) 0-30 min at 1 mg microsomal protein/mL or (b) 5 min at 0 – 2 mg microsomal protein/mL. OH-BDE metabolites were extracted and analyzed by UPLC/MS as described in the Experimental section. Data points are mean ± SEM.

Recovery values for the low and medium QC sample ranged between 84% and 89% for all OH-BDEs. Recovery values for the high QC sample were slightly lower, ranging between 70% and 77% (**Table 4**). The recovery values are higher than those reported in a previous study that used GC-based methods [19], but lower than those reported by Erratico, *et al*. [28] using a similar method to identify the oxidative metabolites of BDE-99. Increasing the extraction volume or the number of extractions, did not increase recovery rates of the OH-PBDE standards.

The method validation studies confirm that OH-BDEs are amenable to direct analysis by UPLC/MS in negative electrospray ion mode without the need for derivatization or extensive fragmentation and that the UPLC/MS-based analytical method that we developed can be used for the separation, detection and quantification of OH-BDEs in a complex biological matrix such as rat liver microsomes.

4.1. Biotransformation of BDE-47 by Rat Liver Microsomes

The validated UPLC/MS method was applied to analyze the oxidative metabolism of BDE-47 *in vitro*. Incubation of BDE-47 with rat hepatic microsomes prepared from PB-treated adult male rats produced five OH-tetra-BDE metabolites (4-OH-BDE-42, 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47 and 4'-OH-BDE-49) which were detected and identified by their retention times and m/z values. The major metabolite was 4'-OH-BDE-49. There was no evidence for the formation of 4'-OH-BDE-17 or 2'- OH-BDE-28 by hepatic microsomes prepared from PB- treated rats. No other metabolite or unidentified peaks were observed.

The effect of varying incubation time (0 - 30 min) and protein concentration (0 - 2 mg/mL) on formation of the five hydroxylated metabolites was investigated using a substrate (BDE-47) concentration of 50 µM. Formation of 4-OH-BDE-42, 3-OH-BDE-47, 5-OH-BDE-47 and 4'-OH-BDE-49 was linear for the first 5 min of incubation at a microsomal protein concentration of 1 mg/mL (**Figure 3a**) and approximately linear up to a microsomal protein concentration of 1 mg/mL at an incubation time of 5 min (**Figure 3b**). Under the experimental conditions used, formation of 6-OH-BDE-47 was detected but could not be quantified because the concentration of this metabolite remained below the LOQ value (10 nM).

5. Conclusions

A UPLC/MS-based analytical method for the separation, detection and quantification of seven possible oxidative metabolites of BDE-47 in rat liver microsomes was developed and validated. The method was applied to analyze the oxidative metabolism of BDE-47 *in vitro* and allowed us to detect formation of five monohydroxylated metabolites of BDE-47 and quantify formation of four of the metabolites. Our method represents an improvement of previously published LC/MS methods because validation was performed using the biological matrix of interest and at the low, medium and high ends of the calibration curve providing a more complete assessment of the accuracy, precision, recovery and LOQ of the analytical method. Quantification of hydroxylated metabolites of BDE-47 generated by hepatic microsomes following a 5 min incubation at 1 mg microsomal protein/mL demonstrates the sensitivity and feasibility of the method for further *in vitro* metabolic studies, including reaction phenotyping, analyzing enzyme-catalyzed reaction kinetics and enzyme inhibition. The selectivity and reproducibility of the UPLC/MS method combined with the *in vitro* metabolism assay will be useful for measuring metabolism of BDE-47 in human liver samples and in liver preparations from additional species.

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